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14. ABSTRACT Our objective is to create a multi-institutional tissue microarray resource from radical prostatectomy samples with detailed clinical information and follow-up and rigorous case-cohort design for use as a platform for validating tissue biomarkers of prognosis. In addition, we have proposed testing a series of biomarkers of prognosis and a set of biomarkers that correlate with Gleason Score. We have made significant progress over the past year. We have completed the tissue microarrays and finalized standard procedures for tissue microarray storage, sectioning and shipping. We have set up a structure for reviewing and approving biomarker proposals based on sound scientific principles and strong preliminary data. We have devised and tested a centralized distribution mechanism at Stanford University of collating and shipping TMAs to participating sites, We have found shortcomings with the BLISS system and STMAD for histological image capture and storage for pathological review and have developed a much improved, highly efficient system using a Leica scanner and Path.XL image analysis software suite. We also have made significant progress in testing TACOMA, an automater TMA scoring algorithm. We have completed staining of the TMAs for H & E, High Molecular Weight Keratin, p27, ERG, SPKINKI, Ki67 (MIBI), MUC1, Survivin and PTEN FISH. Over the next year, we will expand our database to add more tested TMAS Biomarkers, perform QA/QC to ensure high quality, and evaluate their performance for predicting recurrence. We will further refine TACOMA algorithm to facilitate the scoring of TMA stains. We will work with investigators to write papers reporting tested TMA Biomarkers.					
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Validation of Biomarkers for Prostate Cancer Prognosis

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Introduction

As discussed in our progress report last year, the debate surrounding PSA testing has intensified by the final D rating by the US Preventative Services Task Force – stating that PSA testing should not be done because the risks of testing outweigh the benefits. In large part, these recommendations are based on entrenched practice patterns in which nearly all men diagnosed with prostate cancer are treated based on the uncertainty regarding the long-term clinical outcome of men with low and intermediate risk prostate cancer. Standard treatments, mainly surgery and radiation therapy, result in well documented significant morbidities, including significant lower urinary tract symptoms such as incontinence and urinary urgency as well as sexual dysfunction. Furthermore, evidence from many sources suggests that most prostate cancers are relatively indolent, and men will often succumb to other causes of death. However, PSA screening continues to be widely practiced and patients and physicians view the test as better than nothing. Therefore, PSA testing is likely to continue despite USPSTF recommendations. One possible solution to the screening problem is to increase the use of active surveillance (AS) in men with low and very low risk cancers. Acceptance of AS can be enhanced by tests of prognosis that provide some index of risk of the cancer. Recently, Myriad Genetics (Prolaris) and Genomic Health (OncotypeDx Prostate) have introduced gene expression tests that can be performed on biopsies and provide a score of risk. These tools have limited data validating their use in selecting AS. Furthermore, they are very expensive, sometimes cannot be run on small amounts of tissue, and require shipment and processing of biopsies. It is widely recognized that immunohistochemical markers would provide a less expensive assessment of prognosis that could be run on-site and can be run on small amounts of cancer tissue. Unfortunately, at this point, there are few validated immunohistochemical markers of prognosis, although many have been proposed.

To address this challenge, we began our multi-institutional Canary Tissue Microarray Project. We have used rigorous clinical trial case/cohort design, taking care to correct for institutional and spectrum biases. Funding from the Department of Defense allowed us to complete construction of the TMAs as well as the necessary infrastructure and begin testing biomarker candidates. With this infrastructure in place, we now have a robust validation platform for testing prostate cancer biomarkers. We hope and intend that this resource will be a source for future biomarker validation studies even after the DOD funding has ceased. We are pleased to report our progress after 2 years.

Specific Aim 1) To test markers of prognosis on prostate cancer tissue microarrays with associated clinical data.

1.A. Develop work-flow for TMA sharing, image scanning, TMA staining data analysis.

The multi-institutional TMAs have been constructed at all sites. The final TMA cohort is 1326 patients with only 31 patients excluded due to data error. We are in the process of updating follow-up on the TMAs since several years of additional follow-up have been

accumulated since the cases were first selected. Patients have been selected at random from the pool of patients who had undergone radical prostatectomy at each of the sites, with special attention to selecting patients with features typical of low-intermediate risk patients seen in contemporary urologic practices. Details of patient selection, statistical considerations, and TMA construction are summarized in our publication in *Advances in Anatomic Pathology* published earlier this year and appended to last year's report. In addition to this cohort, a separate TMA has been constructed from 220 patients who underwent radical prostatectomy at a sister site who have very long term follow-up (up to 25 years) and hard endpoints including metastases and prostate cancer specific death. Since many of these patients were diagnosed in the pre-and early PSA eras, they are held separately as a validation cohort.

We have completed several stated aims in the proposal with regard to development of work-flow for array sharing, analysis and archiving while some aspects continue to be developed:

- 1) After TMA manufacture was completed, Standard Operating Procedures (SOPs) for TMA storage, sectioning and transferal have all been working well at each site. Staining for the biomarkers currently under evaluation has been universally good, as detailed below.
- 2) In Dr. Brooks Progress Report he detailed the slide shipping, H & E staining, imaging capture and archiving, not reported here.
- 3) One major challenge has been the considerable time required of the pathologists to simply read the TMAs. As mentioned above our TMAs have 1326 patients represented, each with 4 cores. In other words: **1326 pts (x 4 cores) = 5304 cores**. This is a considerable number of cores for the pathologists to read. If they also include H & E and HMWK the work becomes overwhelming, i.e.: **1326 pts (x 4 cores)= 5304 cores (x 3 stains) = 15912 stains**. We are attempting to overcome this with porting into the database the HMWK and H & E staining results so that the pathologists no longer need to look at these while scoring. Regardless, the reading of 5304 cores requires a single pathologist on average approximately 70 hours to look at and score all of the cores. This time commitment is significant, especially considering that the pathologists are not being paid from this or any grant to perform the reads. This has proven to be a major bottleneck in working through candidate biomarkers – yet we have had growing success.

Furthermore, we are attempting to overcome this formidable task of reading TMAs by adding an automated commercial system for reading TMAs that is from Aperio. This scanner allows quantification of colors in a core and can be used for quantitative reads of staining intensity. In addition, the system allows identification of nuclei so that percentage of positive nuclei, in addition to staining intensity, can be collected and quantitated. We have used this system for Ki67 (MIB1) staining and are about to adapt it for p27 staining. Dr. Tim Randolph in our team, in addition to further refine TACOMA algorithm, plans to collaborate with Dr. Richard Levenson at UC Davis to explore the use of some new software that can extract out cancer-related cells from the TMA images, thus reduce the scoring time for pathologists.

4) Data management and data analysis: Dr. Ziding Feng as moved from Biostatistics Program at Fred Hutchinson Cancer Research Institute to Biostatistics Department at MD Anderson Cancer Center, as a professor in Biostatistics, Section Chief in Cancer Early Detection and Biomarker, and Co-Director for a new established Center for Global Cancer Early Detection. Since the clinical database for this project has been completed prior to his moving, the impact on data management is minimal. The MTA has been completed between FHCRC and MDACC so all clinical data for this project have been transferred to MDACC. Each TMA biomarker data, after its completion of the scoring, has been sent to MDACC Dr. Ziding Feng's group. He has start-up funds and supported two statistical analysts Mr. Auston Wei as data analyst and Mr. Aron Joon to manage database. FHCRC has relinquished the grant back to DOD and MDACC has requested the transfer of the grant to MDACC. While the charge on the efforts on the project will be transferred to the DOD grant once the transfer has completed, we do not want to delay the collaborations with Dr. Brooks. All new biomarker data (ERG, Ki67, PTEN) has been analyzed at MDACC, results communicated back to lab investigators, and presented at the face-to-face meeting at Stanford University on October 16, 2013 without any delay. All data analysis results included in this Progress Report were generated at MDACC.

8) Dr. Tim Randolph has led the efforts on adjustments to the TACOMA scoring algorithm in order to evaluate which properties of the TMA images are most important for scoring and accuracy. We have worked with several datasets of TMA images for both refining and evaluating the algorithm, including: 1) the marker CD117 (existing images from Stanford's TMAD); 2) the marker survivin (a proposed Canary study marker); 3) the marker ERG (a proposed Canary study marker). We additionally evaluated two datasets from Drs L True and J Stanford, but after removing samples that were inconsistently scored or contained no stained cancer cells, too few samples remained to obtain a robust estimate of performance. TACOMA's scoring accuracy on CD117 was near 90%, but on survivin and ERG it was 70% or less. Reduced performance is attributable to the substantial heterogeneity of prostate tissue and the identification of cancer cells and we therefore have worked to implement modifications to the algorithm. **As of September 2013, two new Canary Study markers have been scanned and scored and will serve as test sets for algorithm evaluation.**

Algorithm refinements have been aimed at both preprocessing/filtering the tissue images and changes to the transformation that is applied to each image. Specifically, TACOMA is based on the concept of transforming each image into a matrix that summarizes the "co-occurrences" of pixel intensity values, and so we have worked to optimize this transformation, the output of which serves as input to a classification algorithm (random forests). One adjustment we have implemented is to increase the number of co-occurrences calculated per image in order to capture textural patterns at differing scales. In particular, we examined a variety of jump-lengths for computing co-occurrences to see which contribute most to success of the classification algorithm. Additional refinements include a variety of image pre-filtering steps in order to focus the scoring on the most relevant features in the tissue. For the latter, collaboration with pathologists and lab technicians has been valuable.

1.B. Test candidate biomarkers of prognosis for prediction of recurrence after radical prostatectomy

In monthly conference calls, the TMA investigators review progress and review applications for utilizing the TMAP resource. Most applications for use of the TMAs come from within the group, although it is available to the prostate cancer research community broadly and can be accessed by application through the Canary Foundation website (<http://www.canaryfoundation.org>). We have focused on biomarkers that have well characterized, highly performing reagents (e.g. immunohistochemical grade antibodies) and sufficient preliminary data that they could supply prognostic information independent of grade, stage and PSA. We have begun staining for biomarkers listed in our proposal.

1) Proposed biomarkers: We have completed immunohistochemical staining for ERG, SPINK1, p27 (KIP1), MUC1 and Ki67 (MIB1). In all cases, the staining was at exceptionally high quality per initial review of the glass slides by our pathologists. Scores will be correlated with clinical outcome. Since our TMA is uniquely designed for high level validation of markers, we intend to publish finding whether positive or negative so that poorly performing biomarkers can be discarded. In addition to immunohistochemistry, Dr. Jeremy Squire at Queens University, Ontario, Canada has completed FISH to interrogate copy number alterations (allelic loss) at the PTEN locus. The pathologists have completed reads of the slides for PTEN FISH, Ki67 (MIB1), and ERG. These data and their correlation with clinical outcomes are reviewed in Dr. Feng's progress report. We anticipate that each of these biomarkers will result in a high impact publication that we anticipate submitting over the next several months. We are well under way for completing analysis for P27 (KIP1) and SPINK and anticipate these will also be published. Furthermore, we plan to perform an integrated analysis of all biomarkers to generate a model of prognosis.

2) Biomarker analysis results and study finding summary:

Univariate analysis of clinical variables in predicting recurrence: Univariate Cox regression identified the following clinical variables that are predictive for recurrence. They are seminal vesicle invasion (HR=0.30, No vs. Yes, $p<0.0001$), extracapsular invasion (HR=0.69, NO vs. Yes, $p<0.0001$), margin status (HR=1.5, "+" vs. "-", $p<0.0001$), Lymph Node status (HR=5.9, "+" vs. "-", $p<0.0001$), pathological stage (HR=1.7, III/IV vs. I/II, $p<0.0001$), and pre-operative PSA ($p<0.0001$). These analyses provide confidence in the clinical data because they are known factors associated with recurrence after RP.

Univariate analysis of biomarkers in predicting recurrence: PTEN is highly significant in predicting recurrence and exhibit a linear trend (HR=0.62, wild vs. homo-deletion; HR=0.89, hemi-deletion vs. homo-deletion; $p=0.001$). K-M survival curve (Figure 1) demonstrated clear differences among three groups by PTEN deletion status. Ki-67 showed marginal statistical significance (HR=1.03 for one unit increase in %positive Ki-

67, $p=0.09$). Note that the scoring for Ki-67 has not completed and this is based on portion (554/1326) of the data received. ERG is not significantly associated with recurrence.

Multivariate analysis of biomarkers in predicting recurrence: In multivariate Cox regression model pre-operative PSA, prostate weight, margin status, pathology stage were selected into the model and the score calculated from the model was used to plot the Receiver Operating Characteristic (ROC) curve. The area under the ROC curve (AUC) is 0.75. PTEN one variable alone has AUC=0.56. When PTEN is added to the clinical model the AUC did not increase but decreased. This is possible that PTEN deletion is strongly associated with clinical variables and therefore does not add independent contribution in predicting recurrence. Ki-67 alone has AUC=0.59 and when it is combined with clinical model the AUC increased from 0.75 to 0.80. This indicated that Ki-67 has significant independent contribution and improves in predicting recurrence beyond clinical variables.

Specific Aim 2) To evaluate candidate markers that correlate with Gleason grade on prostate cancer tissue microarrays with associated clinical data.

Thus far, we have focused on building the analysis pipeline and in staining high priority biomarkers of prognosis. The intent of this aim is to investigate biomarkers that correlate with Gleason grade. Several markers are in our queue and are listed in the original proposal. For some, we are still looking for high quality affinity reagents that provide interpretable staining with limited background. Leading candidates are AGR2, a marker expressed at high levels in Gleason pattern 3 cancers and Monoamine oxidase A, expressed at high levels in Gleason pattern 4 disease.

For all biomarkers, whether for Gleason score or prognosis, the statistical analysis strategy has been outlined in our proposal and will be used as soon as reads are available from the pathologists.

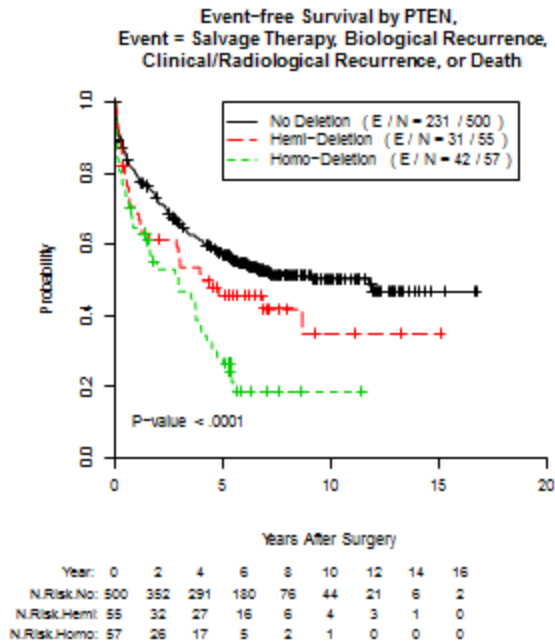


Figure 1: K-M survival curve for three groups by PTEN deletion status

Key Research Accomplishments

- Provided statistical expertise in biomarker review and approval by the investigative team to ensure quality of the reagents and sufficient level of evidence for investigation of a particular biomarker on our valuable resource.
- Data submission and cleaning at Fred Hutchinson Cancer Research Center.
- Porting final clinical data that will be used for analysis of biomarker performance to the MD Anderson DMCC.
- Established and tested the data analysis pipeline for anticipated additional biomarker data.
- Evaluated TACOMA imaging analysis algorithm using Survivin, CD117, and ERG data.
- Preliminary correlation of staining and clinical data for the above biomarkers.
- Validated PTEN and Ki67 in their association and predicting power for recurrence.
- Data suggested that Ki67 is complementary to clinical predictors in predicting recurrence.
- Demonstrated that ERG is not associated with recurrence.

Reportable Outcomes

1) Publications referencing this grant:

James D. Brooks: Translational genomics: The challenge of developing cancer diagnostic biomarkers. *Genome Research* **22**: 183-187, 2012.

Sarah Hawley, Ladan Fazli, Jesse K. McKenney, Jeff Simko, Dean Troyer, Marlo Nicolas, Lisa F. Newcomb, Janet E. Cowan, Luis Crouch, Michelle Ferrari, Javier Hernandez, Antonio Hurtado-Coll, Kyle Kuchinsky, Janet Liew, Rosario Mendez-Meza, Elizabeth Smith, Imelda Tenggarra, Xiaotun Zhang, Peter R. Carroll, June M. Chan, Martin Gleave, Raymond Lance, Daniel W. Lin, Peter S. Nelson, Ian M. Thompson, Ziding Feng, Lawrence D. True and James D. Brooks: Design and construction of a resource for the validation of candidate prognostic biomarkers: the Canary Prostate Cancer Tissue Microarray as a model. *Advances in Anatomic Pathology* **20**: 39-44, 2013.

James D. Brooks: Managing localized prostate cancer in the era of prostate specific antigen testing. *Cancer*, In press, 2013.

Conclusion

We have undertaken a challenging task of creating a multi-institutional TMA resource with rigorous case/cohort design. To our knowledge, such a resource has not been previously created and offers the advantage of reducing institutional biases as well as spectrum biases. In the uniform design and through image acquisition and archiving technologies, we have created a resource that can be easily used by the greater prostate cancer research community. In many ways, this resource represents a gold standard by for evaluation of prognostic biomarkers. We have completed all phases of pipeline construction and continue to refine our work-flow to improve functionality as we work with the resource. We now have tested several biomarkers and confirmed that they are prognostic. We will complete analysis of the biomarkers in the context of the clinical data over the next year and plan several publications. In addition, we will continue to carry out analysis of new biomarkers and solicit applications for biomarkers inside and outside our research group. This research directly addresses the PCRP overarching challenge to *distinguish lethal from indolent disease*.

Request No Cost Extension

The pathologist reading of TMAs has been the bottleneck so we anticipate some biomarker data may come at later stage that the analysis and publication may be beyond the current grant funding period. Also due to Dr. Ziding Feng's move to MDACC, the transfer of the grant will take some time. We have made strategic planning for all these and are committed to complete all planned scope of the work. Therefore, we request one year no cost extension beyond the current grant period to more efficiently use the resources and maximize productivities.